



Screening for stability and compatibility conditions of recombinant human epidermal growth factor for parenteral formulation: Effect of pH, buffers, and excipients

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ABSTRACT

A successful parenteral formulation can be developed by studying stability and compatibility of bio-pharmaceuticals as a function of solution composition. Here, we evaluate the influence of pH, buffers, ionic strength, protein concentration and presence of excipients on recombinant human epidermal growth factor (rhEGF) stability. The stability was accessed by reversed-phase high performance liquid chromatography (RP-HPLC), size exclusion chromatography (SEC-HPLC), enzyme-linked immunosorbent assay (ELISA), Far-UV circular dichroism (CD) and light scattering. The overall maximal stability was obtained in pH near to 7.0 in phosphate, Tris and histidine buffers as the results of the different methods revealed. The CD results revealed that this protein is stable in an extensive pH range. Aggregation of rhEGF was minimized at pH values ranged from 6.0 to 8.0 as indicated the SEC-HPLC and light scattering results. Nor the ionic strength neither the rhEGF concentration had significant effect on the reaction rate constants. Most rhEGF–excipient instability occurs among this protein and reducing sugars. Polymers like poly(ethylene glycol) (PEG) and polysorbates increased methionine oxidation. The rhEGF oxidation and deamidation were the most common degradation pathways. This research identified critical solution factors to be considered for the development of a successful rhEGF parenteral formulation.

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1. Introduction

Human epidermal growth factor (hEGF) is a small polypeptide of 6045 Da, with 53 amino acids residues and six half-cystines that exist in disulfide linkage. Since its discovery by Cohen in 1962 (Cohen, 1962), all aspects of EGF biology have attracted an intense research interest (Wong et al., 2001). In various facets of human health care, the hEGF had shown a potential utility; in particular, in gastric and peptic ulcers (Haedo et al., 1996), corneal epithelial wound (Sheardown and Cheng, 1996), upper respiratory tract (Barrow et al., 1993), wound healing (Brown et al., 1989) and diabetic ulcers among others. The majority of the described EGF applications required the development of different topical (DiBiase

and Rhodes, 1991; Ulubayram et al., 2001), ophthalmic (Kim et al., 2002), and oral formulations (Çelebi et al., 2002; Wong et al., 2001).

Recently, new recombinant hEGF (rhEGF) applications to human health care have been reported. The development of an rhEGF-based cancer vaccine, intended to elicit anti-hEGF antibodies that are able to bind circulating hEGF and prevent its binding to the EGFR (Rodríguez et al., 2010). On the other hand, a new intraleisional injection of rhEGF is under development for the treatment of diabetic foot ulcers (Fernandez-Montequín et al., 2009). In both cases, rhEGF is administered by the parenteral route. For this clinical use, the rhEGF needs to be produced in large quantities by rDNA technology and processes designed to obtain a very pure active ingredient after expression (Wong et al., 2001).

Soluble, extracellular-folded expression of rhEGF in yeast had been reported as an efficient strategy to obtain an rhEGF active pharmaceutical ingredient with high quality and yields (Valdés et al., 2009). The protein is expressed as a mixture of C terminal truncated forms of rhEGF1–51 and rhEGF1–52. Biological activity

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assay of rhEGF1–53, rhEGF1–52 and rhEGF1–51 gave almost identical thymidine uptake dose–response curves. In the same way, the assay of biological activity of different forms of EGF *in vivo* reduced gastric injury with no significant difference between forms (Calnan et al., 2000). On the other hand, the stability of the protein and its compatibility with excipients need to be successfully studied in order to develop stable formulations for parenteral route.

The role of solution environment in protein formulations is of paramount relevance for the chemical and physical protein degradations; among these pH, buffer type and excipients are of great interest (Manning et al., 2010). Biopharmaceuticals are complex products that during drug development require extensive characterization. In that sense, no single analytical method is able to assess every relevant attribute, so a set of physicochemical and biological assay techniques, providing complementary information, are required (Chirino and Mire-Sluis, 2004). The identification of a variable solution factor that contributes to rhEGF stabilization has a great importance to develop stable parenteral formulations.

However, despite the number of reports dealing with rhEGF stability, none of them had been focused on the development of parenteral formulations. For this reason, in this work, we evaluated the influence of pH, buffers, ionic strength, protein concentration and presence of excipients on rhEGF chemical and physical degradation. Excipients compatibility was evaluated by stressing rhEGF at high temperatures and multiple freeze–thawing cycles. Protein stability was accessed by reversed-phase high performance liquid chromatography (RP-HPLC), size exclusion chromatography (SEC-HPLC), enzyme-linked immunosorbent assay (ELISA), Far-UV circular dichroism (CD), light scattering, zeta potential, mass spectrometry (MS) and gel electrophoresis. This study could contribute to the developing of successful rhEGF parenteral formulations.

2. Materials and methods

2.1. Materials

A mixture of the human recombinant EGF1–51 and EGF1–52 expressed in *Saccharomyces cerevisiae* was supplied as concentrated bulk solution by the CIGB (Havana, Cuba). The concentration of protein was determined by UV spectrophotometry at 280 nm using the absorbency value of 30.9 as the extinction coefficient for a 1% (w/v) solution (Carpenter and Cohen, 1979). All chemicals were of analytical grade and excipients met the European Pharmacopoeia (EP). Neutral clear borosilicate glass type I hydrolytic quality vials were acquired from Nuova OMPI (Piombino Dese, Italy), bromobutyl type gray siliconized freeze-drying rubber stoppers were from Helvoet Pharma (Alken, Belgium) and flip-off aluminum seal covered with polypropylene plastic cap were also from Helvoet Pharma (Alken, Belgium).

2.2. pH influence on the rhEGF stability

A concentrated solution of rhEGF, about 5.0 mg/mL was diluted in one step (1:50) to 100 µg/mL. Samples for size-exclusion chromatography HPLC (SEC-HPLC), CD, light scattering and zeta potential were diluted in one step (1:10) to 500 µg/mL. The protein was diluted in 100 mM sodium acetate buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–8.0) and sodium borate buffer (pH 9.0–10.0). Samples were stored at 50 °C and periodically analyzed by RP-HPLC (after 3, 6 and 12 h for pH 9 and 10 and for pH from 3 to 8 samples were also incubated for 1, 2 and 4 days) and SEC-HPLC (after 4 days incubation) to determine the area and purity of rhEGF peaks. The protein concentration was determined by ELISA (Vázquez et al., 1990) after exposure for 2, 4, 12, 15, 21 and 30 days at the same temperature. The effect of pH on the degradation

kinetics of rhEGF was determined as a function of time. For CD, light scattering and zeta potential analysis the samples were freshly prepared.

2.3. Buffer ions influence on the rhEGF stability

A concentrated solution of rhEGF (about 5.0 mg/mL) was diluted in one step (1:50) to 100 µg/mL in a 100 mM buffer solution at different pH values. For pH 6.0, following buffers were studied: sodium acetate, sodium succinate, sodium citrate, sodium phosphate and histidine–HCl, while for pH 7.0 and 8.0 were sodium phosphate, histidine–HCl and Tris–HCl buffers. Samples containing rhEGF with the corresponding buffer pH were dispensed into borosilicate glass vials at 1.0 mL/vial. For stability, samples were stored at 50 °C and periodically analyzed by RP-HPLC (after 1, 3, 5 and 7 days) and ELISA assays (after 7, 15 and 30 days). The kinetics of degradation was determined as a function of buffer pH and type.

2.4. Ionic strength influence on the rhEGF stability

A concentrated solution of rhEGF (about 5.0 mg/mL) was diluted in one step (1:10) to 500 µg/mL in 100 mM sodium phosphate buffer, pH 7.0 containing sodium chloride at different concentration (0, 0.5, 1.0 and 2.0% (w/v)). Then, samples were stored at 50 °C and periodically analyzed by RP-HPLC (after 1, 3, 5 and 7 days), SEC-HPLC (after 7, 14 and 21 and 28 days) and ELISA (after 7, 15 and 30 days). The kinetics of degradation was determined as a function of sodium chloride concentration.

2.5. Protein concentration influence on the rhEGF stability

A concentrated solution of rhEGF (about 5.0 mg/mL) was appropriately diluted to 25, 50, 100, 250, 500 and 1000 µg/mL in 100 mM sodium phosphate buffer, pH 7.0. Then, samples were stored at 50 °C and periodically analyzed by RP-HPLC (after 1, 3, 5 and 7 days) and ELISA (after 7, 15 and 30 days). The kinetics of degradation was determined as a function of protein concentration.

2.6. Excipients influence on the rhEGF stability

A series of 20 excipients were evaluated. For each condition, a placebo was prepared to evaluate interference with analytical methods. The rhEGF was diluted from stock solution (about 5.0 mg/mL), in one step (1:50), to 100 µg/mL in the different excipients solutions. All samples were prepared in 100 mM sodium phosphate buffer, pH 7.0. The excipients concentrations (w/v) were the following: 10% saccharides and polyols, 5% polymers, 0.05% surfactants, 2% amino acids and 1% salts. Samples containing rhEGF with the corresponding excipients were dispensed into borosilicate glass vials at 1.0 mL/vial. For thermal stability, samples were stored at 50 °C and periodically analyzed by RP-HPLC (after 1, 3, 5 and 7 days) and ELISA methods (after 7, 15 and 30 days). The kinetics of degradation was determined as a function of excipients type at 50 °C. Freeze–thawing effects on rhEGF stability were evaluated by RP-HPLC and ELISA assays, in presence of the selected excipients after three cycles of freezing and thawing at –20 °C. Incompatible excipients were also studied in Tris buffer at same pH and concentration.

2.7. Analytical methods

rhEGF preparations were analyzed by RP-HPLC, SEC-HPLC, ELISA, CD, light scattering, zeta potential, MS and SDS-PAGE.

2.7.1. RP-HPLC

The reverse-phase HPLC system (Merck-Hitachi HPLC) consisted of two L-7100 pumps, a D-7455 diode-array detector, a L7350 column oven and a D-700 interface module. rhEGF and its degradation products were eluted from a Vydac C18 column equipped with a Vydac C18 guard column (Vydac, Hesperia, CA) and detected at 226 nm. A linear gradient from 20% B to 40% B was employed over 28 min. Mobile phase A was 0.1% TFA/distilled water and mobile phase B was 0.05% TFA/acetonitrile solution. The injection volume was 300 μ L (corresponding to approximately 30 μ g of rhEGF) and the flow rate was 1.0 mL/min. Relative retention time (RRT) was calculated taking as reference the rhEGF1–52 (RRT of 1.0), in this scale the RRT for rhEGF1–51 was 0.8. Purity was calculated as percentage of the main peak area divided by the sum of the all detected peaks area.

2.7.2. SEC-HPLC

SEC-HPLC was performed using a HPLC system (Merck-Hitachi) consisted of two L-7100 pumps, a D-7455 diode-array detector, a L7350 column oven and a D-700 interface module. A SuperdexTM 75 HR 10/30 column (GE Healthcare Bio-Sciences AB, Sweden) was used with ultraviolet detection at 280 nm and the following chromatography conditions: flow rate 0.4 mL/min, injection volume 200 μ L, run time 55 min. The mobile phase was 100 mM NaPO₄/500 mM arginine-HCl at pH 6.5 (Ejima et al., 2005). Molecular weights were determined using Bio-Rad molecular weight standards (Bovine Thyroglobulin – 670,000 Da, Bovine Gamma Globulin – 158,000 Da, Chicken Ovalbumin – 44,000 Da, Horse Myoglobin – 17,000 Da, Vitamin B-12 – 1350 Da). Multimers, dimer, monomer, and fragments were quantified using the peak absorbance; all amounts are reported as percentage per area.

2.7.3. ELISA

A sandwich-type ELISA was used to quantify the concentration of rhEGF. The rhEGF contained in the sample was captured in an initial step by a monoclonal antibody (mAb, CB-EGF.1) coupled to the solid phase, the second antibody bound to the enzyme (peroxidase-conjugated CB-EGF.2) recognizes rhEGF in a second step. This ELISA procedure was previously described (Vázquez et al., 1990).

2.7.4. Far-UV circular dichroism (CD) and secondary structure

Far-UV (190–250 nm) CD spectra were recorded at Jasco J810 spectropolarimeter (Jasco Inc., Japan), using a 1 mm path length quartz cell. All spectra were recorded after four runs accumulation at 50 μ M rhEGF concentration. Spectra background effects were eliminated after smoothing signal by using FFT (fast Fourier transform) filter and subtracting buffer solution spectra. Secondary structure was predicted by deconvolution of CD spectra using the CONTINLL, CDSSTR and SelCon programs (Provencher and Glöckner, 1981; Sreerama et al., 2000).

2.7.5. Light scattering and zeta potential

Particle size distribution and zeta potential of the samples were carried out on a Zetasizer Nano (Malvern Instruments, UK) in low volume disposable cuvettes at 25 °C. Zeta potential of protein was also measured with the same instrument since it allows determining the electrophoretic mobility to assess the surface electrical charge of particles. For both analyses, each acquisition was performed three times in sample solutions containing 0.5 mg/mL rhEGF in acetate buffer (pH 4.0–5.0) and phosphate buffer (pH 6.0–8.0). DTS applications 5.10 software was used to analyze the data. The hydrodynamic diameter (Dh) of the major species present in solution (evaluated by the volume distribution) was estimated by multimodal analysis of the intensity size distribution.

2.7.6. Mass spectrometry

Mass spectra were obtained in a hybrid orthogonal Q-ToF 2TM tandem mass spectrometer (Micromass, England) equipped with a Z-spray ESI ion source. The capillary and cone voltages were 900 and 35 V, respectively. The TOF analyzer was calibrated using a mixture of sodium and cesium iodides as standard reference. Each collected RP-HPLC fraction was dried in Speed-Vac (Savant, USA) before MS analysis and peptides were dissolved in 60% acetonitrile solution (v/v) containing 0.1% formic acid and directly infused into the MS by using a syringe pump (Harvard Apparatus, USA) at a flow rate of 5 μ L/min.

2.7.7. SDS-PAGE

Samples were directly treated with Laemmli solution and run on a 15% polyacrilamide gel, under reducing conditions (Schägger and von Jagow, 1987). 10 μ g rhEGF was loaded into each lane and detection of resolved protein bands was made by Coomassie Blue staining (Colloidal Coomassie; Invitrogen Ltd., Paisley, UK).

3. Results and discussion

The effects of environmental factors: pH, buffer type, protein concentration and presence of excipients were evaluated on solution stability of rhEGF. High temperature is the most widespread method to degrade therapeutic proteins, which are typically stored under cooled conditions (2–8 °C). With the increase in temperature, proteins may undergo conformational changes which may subsequently lead to other degradation reactions. At the same time, diffusion becomes faster at higher temperature, resulting in more energetic collisions with other protein molecules, as well as with reactive chemicals, thereby favoring both aggregation and chemical degradation reactions. Previous report indicated that, the rhEGF begins to unfold at temperature above 40 °C and have a transition midpoint at 55.5 °C (Yang et al., 2004). The temperature of 50 °C, near to T_m , was chosen to study degradation kinetics in an extreme condition where the protein unfolds is favored. This stressed condition may allow us to select robust stabilization conditions for rhEGF.

3.1. pH effect on stability and aggregation

Impact of pH on rhEGF stability was assessed by RP-HPLC, ELISA, CD, SEC-HPLC and DLS.

Data obtained from experimental runs demonstrated a linear relationship when plots of Ln remaining concentration versus time, indicating pseudo first-order kinetics by both assays: RP-HPLC (Fig. 1A) and ELISA (Fig. 1B). All plots exhibited linearity with correlation coefficients of $r > 0.95$. The pH–rate profiles representing the degradation rates of rhEGF by RP-HPLC and ELISA were obtained by plotting the value of $\log k_{obs}$ against pH as shown in Fig. 1C. The degradation of rhEGF at 50 °C was catalyzed in both acid and basic pH as shown in the results of RP-HPLC and ELISA. The maximum stability of rhEGF was from pH 6.0 to 8.0; with an optimum close to pH 7.0. In a previous stability study of EGF1–48 three main degradation products were characterized by RP-HPLC, Met-21 oxidation, Asn-1 deamidation and the conversion of Asp-11 to a stable Asp-succinimide (Senderoff et al., 1994). We also found three main degradation products in the RP-HPLC chromatograms (data not shown); however, no attempts were made to determine the structure of degradation products and specific degradation pathways for rhEGF at this stage. The rate constant for rhEGF degradation determined by ELISA was significantly smaller than that found by RP-HPLC (Fig. 1C). It is well recognized that mAb-based ELISA assay is less sensitive to simultaneously detection of different chemical modifications as RP-HPLC probably does. On the other hand, this

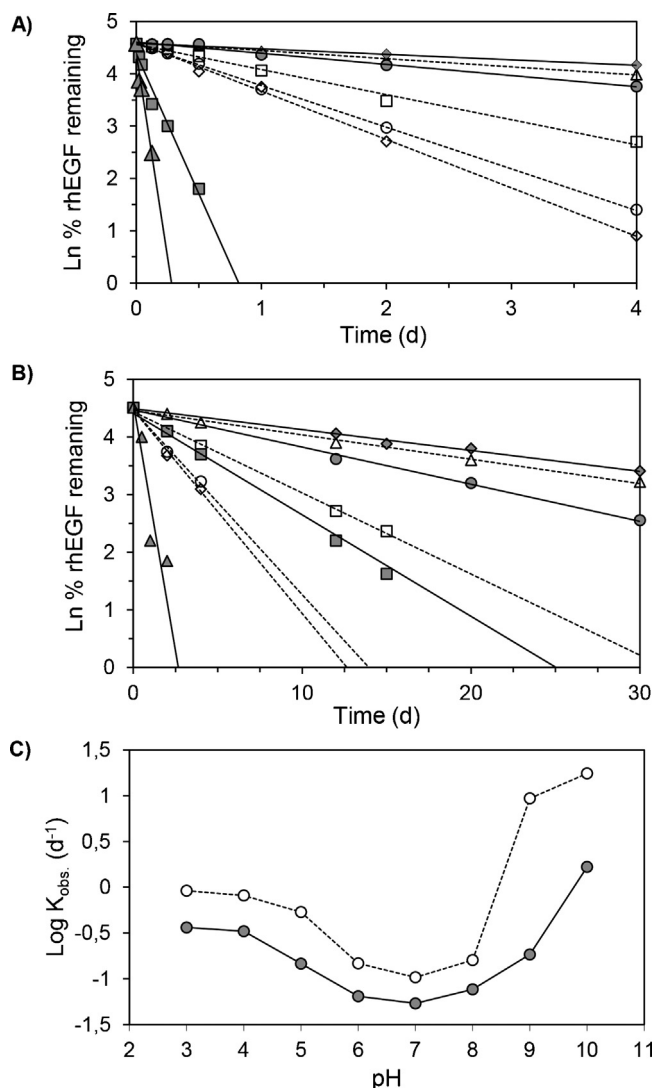


Fig. 1. Solution pH effect on degradation rate profile for rhEGF at 50 °C by RP-HPLC (A) and ELISA (B). Acid pH dashed lines and open symbols: pH 3.0 (◇), pH 4.0 (○), pH 5.0 (□), pH 6.0 (△); neutral and basic pH continuous line and closed symbols: pH 7.0 (◆), pH 8.0 (●), pH 9.0 (■), pH 10.0 (▲). (C) pH-rate profile obtained by both methods: ELISA dashed lines and open circles (○) and RP-HPLC continuous line and closed circles (●).

mAb-based ELISA assay is potentially sensitive to detect proteins conformational changes (Puchades et al., 2005).

The secondary structure of rhEGF was studied at far UV region as a function of pH (Fig. 2). All CD spectra, in both basic and acidic conditions, showed a profile characteristic of folded forms. This assumption was verified when the amount of secondary structure was calculated from the CD spectrum resulting in a high content of random structures (64%), which comprises turns (24%), unordered forms (33%) and a minor content of distorted helical (7%). It was also found a content of beta structures (36%) and no regular helix, all consistent with a CD negative minimum around 200 nm. Better results were achieved from CONTINLL program, with a root mean square difference between experimental and calculated curves (RMSDExp-Calc) lower than 2% for all deconvolutions. Besides that, one can notice only a small variation around 200 nm (202–204 nm), when compared pH 3.6, 5.6, 6.0 and 8.0 to pH 7.0 (native one). In spite of this variation, all spectra pointed to a very similar secondary fold, mainly constituted by random forms, as can be found in native form. These results revealed that rhEGF is stable in an extensive range of pH, keeping the secondary fold very similar

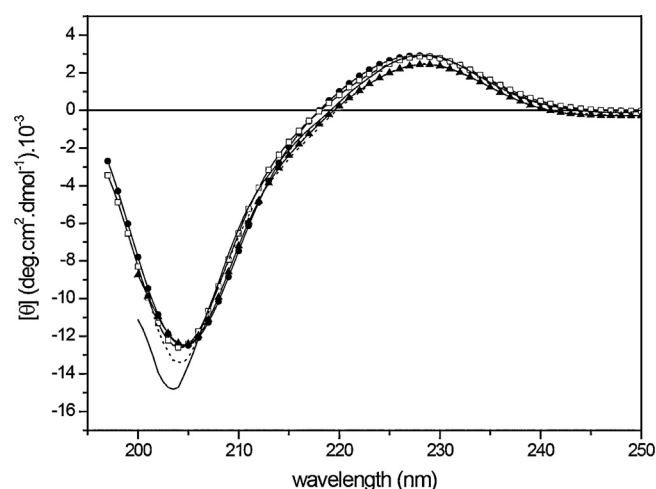


Fig. 2. Protein circular dichroism spectra of rhEGF at pH 3.6 (---), pH 5.6 (▲), pH 6.0 (□), pH 7.0 (—) and pH 8.0 (●).

to the native one, except for rhEGF at pH 4.6, where the samples became fully precipitated. Holladay et al. (1976) showed a very similar CD spectra for native mEGF; with a shoulder around 220 nm (by intramolecular disulfide bonds and aromatic residues) and a spectra shape expected for random forms and β-sheets. Altogether, these statements pointed toward a native fold for rhEGF.

The effect of solution pH on aggregation behavior of rhEGF was followed by size exclusion chromatography (SEC) and dynamic light scattering (DLS). To describe the aggregation state of the rhEGF under different pH values, we show in Fig. 3A the size exclusion chromatograms of 0.5 mg/mL protein solution after an incubation time of 4 days in 100 mM sodium acetate (pH 3.0–5.0), sodium phosphate (pH 6.0–8.0) and sodium borate (pH 9.0–10.0) buffers at temperature of 50 °C. The peak at about 37.5 min elution time corresponds to the rhEGF monomer with a molecular weight of 6190 g/mol, as derived from the calibration curve (Fig. 3A). The peaks at shorter elution times represent aggregates, while the peaks at later elution times represent clipped rhEGF variants. Three types of behavior can be identified in the pH range investigated in Fig. 3A after 4 days of incubation time at 50 °C: (1) stable solutions at pH range from 6.0 to 8.0 which exhibit the same SEC chromatogram of the non-stressed sample with purity and mass recovery higher than 93.9%; (2) formation of soluble oligomers at pH range from 9.0 to 10.0 with significant reduction in purity and mass recovery (from 50.1% to 85.5%), and (3) formation of rhEGF clipped variants and significant mass lost recovery (from 50.1% to 85.5%) in the pH range from 3.0 to 5.0. Aggregation and mass loss were promoted by increasing pH while clipping and mass loss by decreasing pH.

It is well known that the off-line SEC analysis suffers from limitations due to interactions between the aggregates and the stationary phase of the column or possible formation of monomer from reversible aggregates upon dilution with the mobile phase (Carpenter et al., 2010). It is therefore important to check the reliability of the SEC chromatograms by performing comparison with orthogonal techniques (Carpenter et al., 2010). For this purpose, samples in the pH range from 4.0 to 8.0 were also analyzed by DLS; this technique can also provide good information on aggregate size. The DLS results, normalized by the volume of the scattering particle, from rhEGF samples in the pH range from 4.0 to 5.0 showed that the majority of proteins were in very large size aggregation state (Fig. 3B). Otherwise, the DLS results from the samples in the pH range from 6.0 to 8.0 revealed that the major species were particles in the size ranging from 2 to 3 nm, which can be attributed to a monomeric structure. A clear shift in the population toward HMW

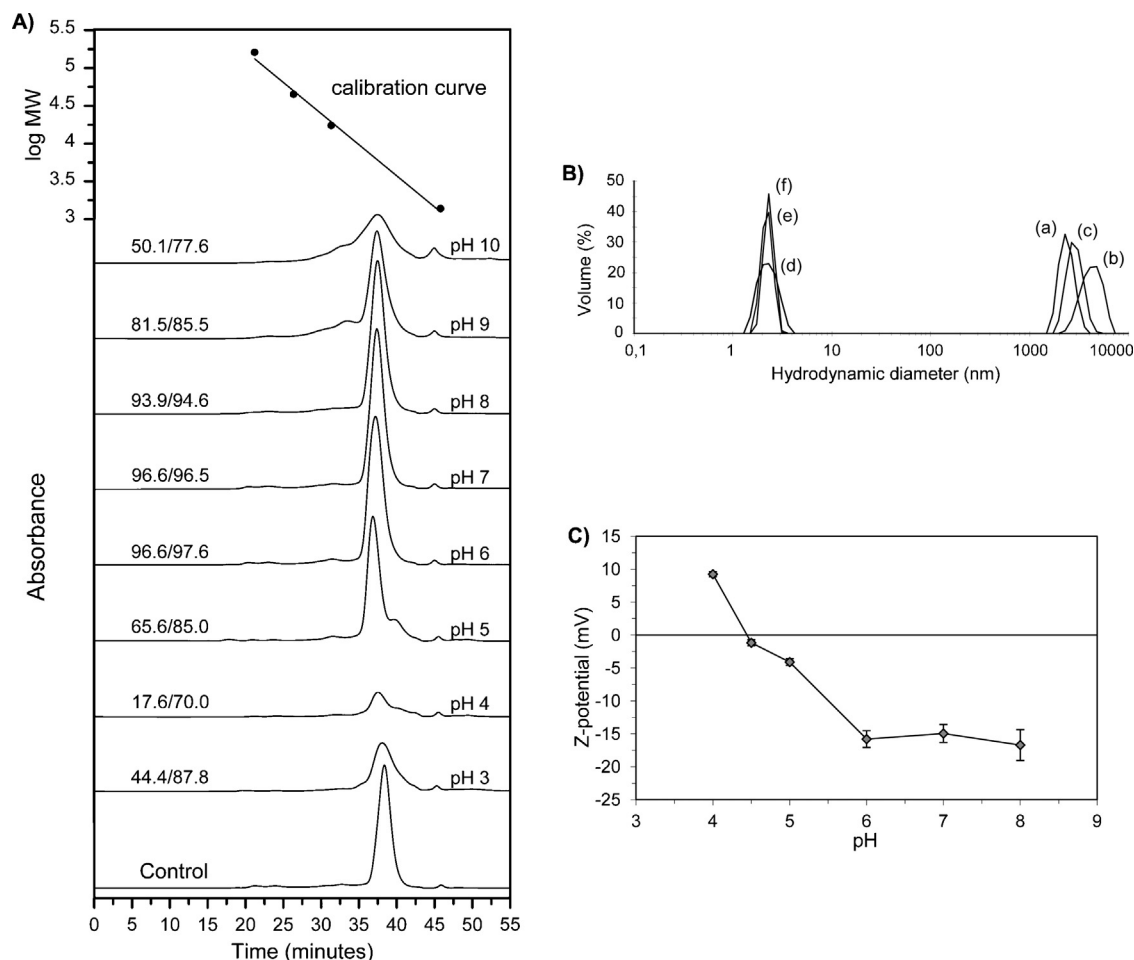


Fig. 3. Effect of pH on rhEGF aggregation and charge. (A) SEC chromatograms of 0.5 mg/mL protein solution after 4 days of incubation at 50 °C and several pH, chromatograms also shows recovery/purity data. (B) Volume-weighted size distributions by DLS of 0.5 mg/mL rhEGF at various pH: (a) pH 4.0, (b) pH 4.5, (c) pH 5.0, (d) pH 6.0, (e) pH 7.0, and (f) pH 8.0. (C) pH dependence of the zeta potential. The recovery (%) of rhEGF by SEC-HPLC was calculated as the rate of main peak area after 4 days of incubation at 50 °C to the main peak area of the control sample (untreated) and multiplies by 100.

species was detected below pH 6.0. The results obtained by SEC and the non-fractionated samples evaluated in situ by DLS were found to be reasonably in agreement.

In addition, the same sets of samples measured by DLS were also studied by zeta potential (Fig. 3C). The zeta potential for pH 4.5 was very near to zero, this behavior was consistent with the previously reported isoelectric point near to 4.6 for this protein (Senderoff et al., 1994). On the other hand, the zeta potential of rhEGF was positive for pH below 4.5 and negative above such pH value. The zeta potential from samples with pH higher or equal to 6.0 was of larger values in comparison with the pH below 6.0. The lower zeta potential values strongly correlated with the presence in solution of aggregate species of HMW; and the larger zeta potential values with the presence in solution of particles in the size ranging from 2 to 3 nm that can be attributed to the rhEGF monomer (Fig. 3B and C). These results suggest that the aggregation process in the pH range from 4.0 to 5.0 was probably mediated by electrostatic interactions (Roberts et al., 2011). Control of protein aggregation is an ubiquitous concern during purification, formulation and manufacture of therapeutic proteins (Roberts et al., 2011). The protein solution pH is considered one of the most important factors to control protein aggregation (Wang, 2005).

Summarizing, the solution pH range from 6.0 to 8.0 was selected for future studies because it favors the chemical, conformational and physical stability of rhEGF as these RP-HPLC, ELISA, CD, SEC-HPLC, light scattering and zeta potential results indicate.

3.2. Buffer type effect

Buffer catalysis had been identified as a main factor in the solution stability studies of many proteins and peptides (Bell, 1997). The degradation of rhEGF was also studied in aqueous solution at the maximal stability pH values from 6.0 to 8.0 in different buffer ions at 50 °C. The rate constants were obtained from the slopes of the semilog plot of remaining concentration versus time by statistical regression analysis. The observed reaction first-order rate constants of rhEGF are listed in Table 1.

At pH 6.0 the buffer solution containing histidine ion showed a remarkable increase in their degradation rate in comparison to others buffers by both assays. At this pH value, the lower reaction rate constants were obtained in succinate and citrate buffers by RP-HPLC, while the lower by ELISA were in succinate, acetate and phosphate buffers. At pH 7.0 the solutions containing phosphate and Tris buffers showed the lower reaction rate constants by both assay. At pH 8.0 the lower observed reaction rate constants attained by RP-HPLC were obtained in histidine and Tris buffers, but in the ELISA the lower were in Tris and phosphate buffers. In general, the rhEGF was proven to be more stable at pH near to 7.0 in phosphate and Tris buffers.

The extent to which a particular protein may be stabilized or destabilized by a combination of pH and buffer depends on many factors (Ugwu and Apte, 2004). One influencing factor could be the interactions among ionizable groups (pK_a) of the buffers and the

Table 1

Kinetic parameters for rhEGF thermal stability at 50 °C, as a function of pH and buffers. Data expressed as individual data points.

pH	Buffer type	k_{obs} ($\times 10^{-2}$ day $^{-1}$)						$T_{1/2}$ (day) ^a					
		Area ^b		Purity ^b		ELISA		Area ^b		Purity ^b		ELISA	
6.0	Acetate	13.9	14.9	13.6	14.6	5.8	6.6	4.7	5.0	4.7	5.1	9.4	11.9
	Succinate	12.7	14.4	10.1	11.5	5.6	6.6	4.8	5.5	6.0	6.9	9.1	12.4
	Citrate	12.1	13.3	11.5	12.5	8.5	9.4	5.2	5.7	5.5	6.0	6.7	8.2
	Phosphate	13.6	15.2	14.2	15.8	7.5	8.5	4.6	5.1	4.4	4.9	8.2	10.7
	Histidine	23.4	24.6	22.2	23.4	8.9	10.7	2.8	3.0	3.0	3.1	5.5	7.8
7.0	Phosphate	9.8	10.4	9.1	9.7	6.4	7.1	6.7	7.1	7.1	7.6	9.8	12.2
	Histidine	10.0	14.8	9.2	10.2	6.7	7.7	4.7	6.9	6.8	7.5	8.0	10.3
	Tris	8.2	11.0	8.1	9.1	5.6	6.5	6.3	8.5	7.6	8.6	9.4	12.4
8.0	Phosphate	13.5	14.7	9.0	9.8	6.4	7.0	4.7	5.1	7.1	7.7	9.1	10.8
	Histidine	11.1	11.9	9.0	9.7	6.7	7.7	5.8	6.2	7.2	7.7	7.9	10.4
	Tris	11.1	12.4	10.6	11.2	7.7	8.7	5.6	6.3	5.8	6.6	7.1	9.0

^a $T_{1/2}$ (day) = $0.693/k_{\text{obs}}$ (day $^{-1}$).^b Results obtained by RP-HPLC analysis.

side chain of protein amino acids (Ugwu and Apte, 2004). The difference in stability of rhEGF to variations in pH and buffers could be the results of many factors.

Although phosphate and Tris pH 7.0 buffers showed similar results, due to the following reason phosphate was selected for further studies. First, at pH 7.0, Tris buffer has a lower buffering capacity than phosphate (pK_a of Tris = 8.06 and pK_a of phosphate = 7.20). Second, Tris buffer possesses relatively high $\delta pK_a/\delta T$ values ($-0.028/^\circ\text{C}$) when compared with phosphate ($-0.0028/^\circ\text{C}$ value) (Beynan and Easterby, 1996). Furthermore, phosphate buffer is economically cheaper than Tris and also more widely used in protein formulations.

3.3. Effect of ionic strength

Ionic strength is considered a main factor that modulated colloidal stability and protein aggregation by electrostatic interactions and also for protein deamidation (Manning et al., 2010). The modulation of ionic strength by sodium chloride concentration is also a mean to adjust osmolality of parenteral formulations. The effect of ionic strength on rhEGF stability was investigated at 50 °C, in 100 mM phosphate buffer pH 7.0; by RP-HPLC, SEC-HPLC and ELISA. The rate constants were obtained from the slopes of the semilog plot of remaining concentration versus time by statistical regression analysis. The observed reaction first-order rate constants of rhEGF are listed in Table 2. The rhEGF stability, by the different methods studied, showed similar rate constants for the different sodium chloride concentration. Among the different methods the higher rate constants were obtained by RP-HPLC followed by ELISA and then by SEC-HPLC. As shown in Fig. 4, the aggregation of rhEGF as determined by SEC-HPLC, was not affected as ionic strength change with sodium chloride concentration under the studied conditions. It is very notorious the high stability against aggregation at high temperature of rhEGF as determined by SEC-HPLC near to physiological pH (Fig. 4).

3.4. Effect of protein concentration

Protein concentration has been identified as a main factor in the solution stability of many proteins and peptides (Frokjaer and Otzen, 2005; Ross and Shrake, 1988; Wang, 2005). The effect of rhEGF protein concentration showed the value of purity and ELISA concentration decreased throughout the study due to the storage condition, 50 °C (Fig. 5). The kinetic analysis showed a higher capability of RP-HPLC with respect to the ELISA assay to detect the degradation of rhEGF. A small increase in the reaction rate could be supposed from the k_{obs} by RP-HPLC area and ELISA, at 25 $\mu\text{g/mL}$.

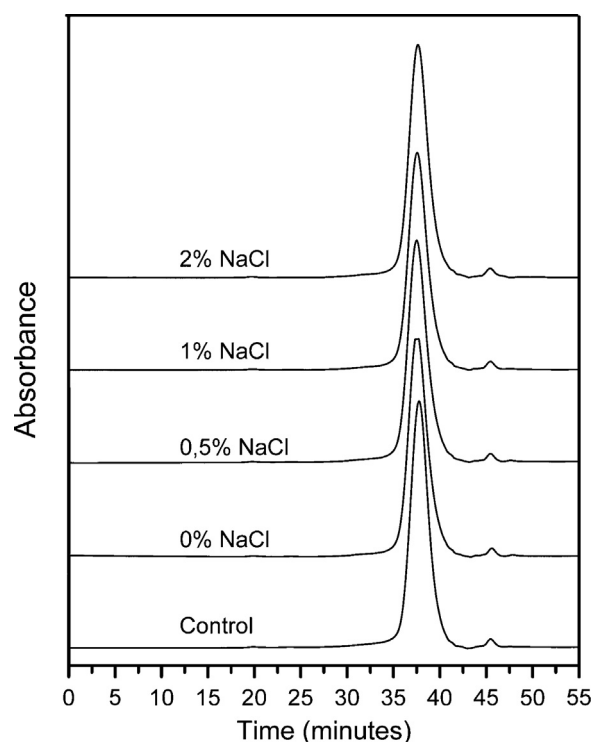


Fig. 4. Effect of ionic strength, adjusted with sodium chloride, on rhEGF aggregation by SEC-HPLC after 21 days of exposure at 50 °C.

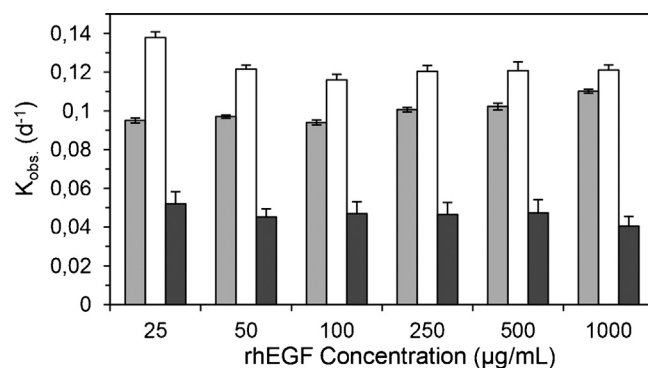


Fig. 5. Effect of protein concentration on rhEGF stability by RP-HPLC and ELISA. Purity by RP-HPLC (gray bar), Area by RP-HPLC (open bar), and ELISA (black bar).

Table 2

Kinetic parameters for rhEGF thermal stability at 50 °C, as a function of NaCl concentration. Data expressed as individual data points.

NaCl (%)	$k_{\text{obs}} (\times 10^{-2} \text{ day}^{-1})$								$T_{1/2} (\text{day})^a$							
	RP-Area ^b		RP-Purity ^b		SEC-Area		ELISA		RP-Area ^b		RP-Purity ^b		SEC-Area		ELISA	
0	9.2	10.6	7.8	9.1	0.53	0.68	4.6	6.3	6.5	7.5	7.6	8.9	101.9	130.8	11.0	15.1
0.5	9.4	10.3	8.2	9.2	0.42	0.56	6.0	7.8	6.7	7.4	7.5	8.5	123.8	165.0	8.9	11.6
1.0	9.0	10.2	8.2	9.2	0.55	0.72	5.4	6.3	6.8	7.7	7.5	8.5	96.3	126.0	11.0	12.8
2.0	9.5	10.7	7.8	9.0	0.43	0.54	3.8	5.0	6.5	7.3	7.7	8.9	128.3	161.2	13.9	18.2

^a $T_{1/2} (\text{day}) = 0.693/k_{\text{obs}} (\text{day}^{-1})$.^b Results obtained by RP-HPLC analysis.

A possible explanation to the increase in rate constant at the lowest protein concentration could involve the adsorption of rhEGF to the wall of glass vials, as previously described by Jørgensen et al. (1999). On the other hand, a small increase in the reaction rate could be observed from the k_{obs} by RP-HPLC purity at 1000 µg/mL. It has been suggested that a high concentration may facilitate the aggregation of proteins (Ross and Shrake, 1988). This influence can be explained due to the increment on intermolecular collisions, which may increase the reaction rate degradations on these molecules. These results indicate that the rhEGF concentration had no significant effect on the observed pseudo first-order reaction rate in the investigated range.

3.5. Excipients compatibility

The objective of this study was to identify the ability of some excipients to perform a beneficial or a detrimental effect on rhEGF stability. Commonly used stressed conditions against protein stability, like high temperature and freeze and thawing cycles, were selected to evaluate the performance of various excipients. The selection of 20 excipients (sugars, polyols, amino acids, polymers, surfactants and salts) and their concentration was based on their presence in marketed drug products for parenteral use. The excipients were evaluated because they demonstrated effects on protein stabilization (Manning et al., 2010; Wang, 2000): saccharides/polyols, amino acids, surfactants, polymers and salts.

The excipient compatibility study was performed in sodium phosphate by using analytical stability indicative methods. RP-HPLC method is very sensitive to detect chemical incompatibilities of drugs with excipients (Thomas and Naath, 2008; Wu et al., 2009) and the mAb-base ELISA has the ability to detect conformational changes on rhEGF because of one mAb used in this ELISA recognized a topographical epitope on rhEGF (Puchades et al., 2005). It was also verified that the excipients did not interfere with analytical methods.

3.5.1. Thermal stability

Accelerated aging is one of the most appropriate methods of rapidly and accurately assessing the chemical stability of pharmaceutical dosage forms. This method is appropriate to determine oxidation, hydrolysis, reaction with reactive excipient impurities and protein denaturation (Kenneth and Roger, 2005).

The evaluation of selected excipients on rhEGF stability stressed at high temperature revealed the incompatibility of three saccharides, one polymer and one salt (Table 3). The reducing sugars (lactose, glucose and fructose) affected rhEGF stability after stressed at high temperature by both assays (RP-HPLC and ELISA). Protein modification by reducing sugars is already described in literature by the called browning reaction (Paulsen and Pflughaupt, 1980). Reducing sugars can react with lysine and arginine residues in proteins to form carbohydrate adduct via the Maillard reaction (Chuyen, 1998). The polyethylene glycol (PEG) 6000 was the other excipient, which affected the rhEGF stability by RP-HPLC. Protein oxidation by peroxide traces in PEG excipients is already described

in literature (Kumar and Kalonia, 2006). The stability of rhEGF in presence of PEG 6000 was not affected in the ELISA assay. We demonstrated that this mAb-based ELISA is not sensitive to the Met-21 oxidation on rhEGF (data not shown).

The rhEGF solution in presence of MgCl_2 changes the clear aspect to an opalescence solution in phosphate buffer (data not shown). This excipient affected the rhEGF stability by RP-HPLC but not by ELISA. A possible explanation to the difference between both assays could be due to the fact that prior to RP-HPLC analysis the sample is centrifuged, but not by ELISA. During the sample dilution by ELISA assay the precipitate may be re-dissolved. Phosphates form insoluble salts with bivalent metals, like Mg^{2+} , and precipitate (Wazer and Callis, 1958). The addition of MgCl_2 to the rhEGF solution in presence of phosphate would induce the precipitate formation at high temperature. Protein sequestration by the precipitate could be the cause of the affected RP-HPLC results.

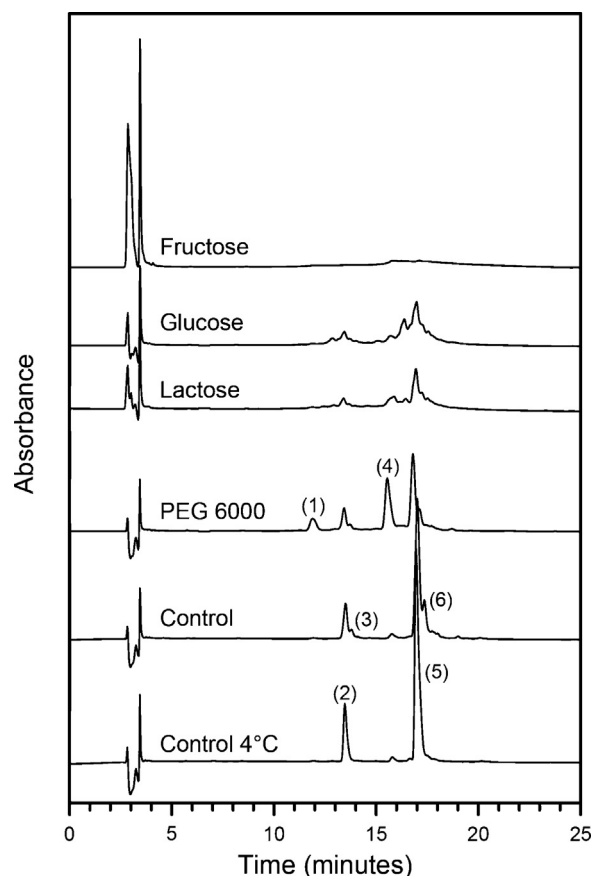


Fig. 6. RP-HPLC chromatographic profiles for rhEGF, after heating by 3 days at 50 °C, in 100 mM sodium phosphate buffer pH 7.0 containing incompatible excipients. Peak (1): early eluting rhEGF1-51 by-product; peak (2): native rhEGF1-51; peak (3): later eluting rhEGF1-51 by-product; peak (4): early eluting rhEGF1-52 by-product; peak (5): native rhEGF1-52; peak (6): later eluting rhEGF1-52 by-product.

Table 3

Kinetic parameters for hEGF thermal stability at 50 °C, as a function of excipient type. Data expressed as individual data points.

Group	Excipients	k_{obs} ($\times 10^{-2}$ day $^{-1}$)						$T_{1/2}$ (day) ^a					
		Area ^b		Purity ^b		ELISA		Area ^b		Purity ^b		ELISA	
Control	–	9.0	10.4	8.4	9.6	5.0	6.6	6.7	7.7	7.2	8.3	10.5	13.9
	Sucrose	10.6	11.4	9.7	10.4	3.7	4.9	6.1	6.5	6.7	7.2	14.1	18.7
Saccharides	Trehalose	11.4	12.7	10.7	11.4	5.6	6.9	5.5	6.1	6.1	6.5	10.1	12.5
	Rafinose	11.5	12.5	11.5	12.6	4.0	5.7	5.5	6.0	5.5	6.1	12.3	17.5
	Lactose	31.4	34.6	28.5	31.5	12.4	16.1	2.0	2.2	2.2	2.4	4.3	5.6
	Glucose	27.4	28.6	24.3	25.7	11.5	15.3	2.4	2.5	2.7	2.9	4.5	6.0
	Fructose	110.0	114.1	83.9	88.1	27.9	34.5	0.6	0.6	0.8	0.8	2.0	2.5
Polyols	Manitol	9.7	10.4	9.6	10.4	4.4	5.7	6.7	7.2	6.7	7.2	12.3	15.9
	Sorbitol	11.2	12.8	10.3	11.7	5.1	6.7	5.4	6.2	5.9	6.7	10.3	13.6
	Glycerol	10.5	11.5	10.5	11.5	7.9	9.6	6.0	6.6	6.0	6.6	7.3	8.8
Aminoacids	Glycine	12.3	13.7	10.4	11.6	5.4	7.0	5.1	5.6	6.0	6.7	9.9	12.8
	Alanine	11.7	12.3	10.7	11.3	5.8	7.4	5.6	5.9	6.1	6.5	9.4	11.9
	Leucine	10.7	11.4	9.7	10.3	5.4	7.4	6.1	6.5	6.7	7.1	9.4	12.8
	Histidine	10.4	11.6	10.4	11.6	4.6	6.6	6.0	6.7	6.0	6.7	10.5	15.1
Surfactants	Polysorbate 20	11.3	12.7	10.4	11.7	5.2	6.8	5.5	6.1	5.9	6.7	10.2	13.3
	Polysorbate 80	10.6	11.5	10.6	11.4	4.9	6.1	6.1	6.6	6.1	6.5	11.4	14.1
Polymers	Dextran-40	12.2	13.9	11.4	12.6	6.0	7.7	5.0	5.7	5.5	6.1	9.1	11.6
	PEG 6000	21.0	23.1	19.0	21.0	3.9	5.3	3.0	3.3	3.3	3.6	13.1	17.8
Salts	NaCl	9.7	10.4	7.7	8.3	3.7	4.9	6.7	7.2	8.3	9.0	14.1	18.7
	KCl	9.7	10.4	8.7	9.4	4.8	6.1	6.7	7.2	7.4	8.0	11.5	14.6
	MgCl₂	19.4	20.6	18.5	19.5	6.9	8.2	3.4	3.6	3.6	3.7	8.5	10.1
Incompatible excipients in Tris buffer	–	9.4	10.0	9.6	10.0	4.8	6.2	6.9	7.4	6.9	7.2	11.2	14.5
	Lactose	16.5	17.7	14.9	15.1	6.0	7.8	3.9	4.2	4.6	4.7	8.8	11.5
	Glucose	19.6	20.7	15.2	15.6	7.2	8.9	3.3	3.5	4.4	4.6	7.8	9.6
	Fructose	29.4	30.8	19.2	22.3	9.6	11.3	2.3	2.4	3.1	3.6	6.1	7.2
	PEG 6000	73.0	76.3	68.5	70.0	11.7	13.7	0.9	1.0	1.0	1.0	5.1	5.9
	MgCl ₂	9.3	9.7	8.8	9.6	3.6	4.8	7.1	7.5	7.7	7.9	14.4	19.3

Incompatible excipients are in bold.

^a $T_{1/2}$ (day) = $0.693/k_{\text{obs}}$ (day $^{-1}$).^b Results obtained by RP-HPLC analysis.

Because sodium phosphate was selected for the compatibility study, to convolute the effect of buffer species versus incompatible excipients, Tris buffer was included as comparator to understand if the effects at same pH were related to excipient or to buffer species. The reducing sugars (lactose, glucose and fructose) affected rhEGF stability after stressed at high temperature by both assays (RP-HPLC and ELISA) in both buffers, but the rhEGF was more affected in phosphate than in Tris buffer (see Table 3). In the case of PEG, the buffer effect was contrary; the rhEGF was more affected in Tris than in phosphate buffer (see Table 3). In the case of MgCl₂ in Tris buffer, no changes in the solution appearance were observed. Contrary to phosphate buffer, MgCl₂ in Tris buffer does not affected the rhEGF stability either by RP-HPLC or by ELISA. The results indicate an interaction effect among excipients and buffer ions. Nevertheless, most of the excipients that exert a detrimental effect on rhEGF stability in phosphate buffer also were detrimental in Tris buffer.

Representative RP-HPLC chromatograms of the most significant incompatible excipients on rhEGF stability at high temperature are shown in Fig. 6. The untreated control sample represents the two rhEGF isomers rhEGF1–51 (peak 2) and rhEGF1–52 (peak 5). The control sample after heating by three days at 50 °C showed an increase in the peaks 3 and 6. These peaks were characterized by mass spectrometry with the purpose of identifying these modifications at molecular level (see Table 4). Peaks 3 and 6 were identified as rhEGF modified at Asp-1 by deamidation. The sample containing PEG 6000 showed a strong additional increase in peaks 1 and 4; those peaks correspond to Met-21 oxidation for rhEGF1–51 and rhEGF1–52 respectively (see Table 4).

The RP-HPLC profile for the reducing sugars (lactose, glucose and fructose) display a strong reduction on the peaks corresponding to rhEGF1–51 and rhEGF1–52. These chromatograms also represent

a simultaneous broadening of the main peaks and appearance of multiple new peaks. The fructose was the most affected sample, with no signal detected in their chromatogram. These behaviors on RP-HPLC profiles are probably due to the interaction of such sugars with rhEGF protein and the formation of the corresponding glycation products (Rošćić and Horvat, 2006). The gel electrophoresis analysis showed important differences in rhEGF migration patterns, comparing to control, Fig. 7, indicating an apparent protein glycation (Ledesma-Osuna et al., 2008). In this sense, rhEGF in the presence of lactose, glucose or fructose showed an electrophoreses migration pattern broader and slower than the one of untreated rhEGF, which may indicate that glycosylated samples could contain a range of protein molecules with different number of coupled sugar

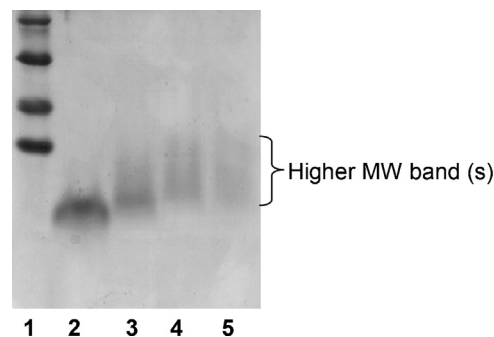


Fig. 7. Reducing sugars effect on the SDS-PAGE profile for rhEGF solution after 30 days at 50 °C. Lane (1): molecular weight (MW) standard markers (lysozyme (14.3 kDa), b-lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43.5 kDa) and bovine serum albumin (67)); lane (2): control at 4 °C; lane (3): lactose; lane (4): glucose and lane (5): fructose.

Table 4
Identification of the main degradation products of rhEGF by RP-HPLC/ESI-MS.

Peak number	RRT ^a	m/z theor	m/z exp	Dif (Da)	Identification
1	0.71	5962.66	5962.53	0.13	rhEGF1–51 oxidation (at Met-21)
2	0.80	5946.66	5946.70	0.04	Authentic rhEGF1–51
3	0.82	5947.64	5947.69	0.05	rhEGF1–51 deamidation (at Asn-1)
4	0.93	6075.82	6075.91	0.09	rhEGF1–52 oxidation (at Met-21)
5	1.00	6059.82	6059.67	0.15	Authentic rhEGF1–52
6	1.02	6060.80	6060.67	0.13	rhEGF1–52 deamidation (at Asn-1)

^a Relative retention time (RRT) = [(retention time of modified rhEGF)/(retention time of authentic rhEGF1–52)].

residues. Furthermore, rhEGF in the presence of lactose presented tighter bands than rhEGF with monosaccharides. These findings suggest that fructose and glucose were more reactive than lactose.

None of the excipients studied showed a significant stabilizing effect on rhEGF, comparing to control (Table 3). Further, as shown in Fig. 6 for control sample after heating at 50 °C, the peak corresponding to rhEGF deamidation (Table 4) was consistently increased independent of the presence or absence of the excipients (data not shown). Consequently, this result suggests the difficulty to achieve a long-time stable rhEGF liquid formulation for parenteral route.

3.5.2. Freeze–thawing effect

Freeze–thawing experiments are useful to identify excipients that stabilize during freezing (also a step in lyophilization) or frozen storage. During freezing and thawing, proteins are exposed to a combination of stress factors: interfacial stresses, temperature fluctuations, cryoconcentration, crystallization of excipients, phase separation and pH shifts (Bhatnagar et al., 2007).

The results of the evaluation of this stress on rhEGF compatibility with excipients are shown in Table 5. The saccharides type excipients affected rhEGF stability by the RP-HPLC assay in the

samples of the reducing sugars group. The non-reducing sugar sucrose and trehalose showed the better protective effect in both assays. Polyols manitol, sorbitol and glycerol showed good stabilizing effect on RP-HPLC stability of rhEGF after freeze–thawing. In general, the effects of amino acids and salts on rhEGF stability were not very different from the control sample (not excipient). The surfactants (polysorbate 20 and 80) and PEG 6000 affected the rhEGF stability after the freeze–thawing cycles by RP-HPLC purity assay. The most common degradation pathway of rhEGF during the freeze–thawing stress was the increase in the peaks 1 and 4 (Fig. 8) corresponding with Met-21 oxidation (Table 4).

Because phosphate buffer may have pH change during the freezing and thawing process, excipients that affected the rhEGF stability during this stress were also evaluated in Tris buffer as control. As shown in Table 5, among the excipients that affected the rhEGF stability in phosphate buffer (fructose, polysorbates and PEG 6000) by RP-HPLC, just the polysorbates also affected rhEGF stability in Tris buffer in similar way as in phosphate. The more sensitivity of rhEGF to be affected by fructose and PEG in phosphate may be due to a decrease in pH during the freeze–thawing process (Murase and Franks, 1989), or due to an interaction factor of these excipients with the buffer ions. Under freeze–thawing process,

Table 5
Effect of different excipients on rhEGF stability after three cycles of freezing and thawing at –20 °C. Data expressed as individual data points.

Group	Excipients	Area (%) ^a	Purity (%) ^b	ELISA (%) ^c
Control	–	100.1	93.2	85.0
Saccharides	Sucrose	96.9	95.2	100.4
	Trehalose	96.4	95.7	80.7
	Rafinose	91.3	93.6	95.2
	Lactose	93.0	92.4	98.5
	Glucose	93.7	95.1	97.7
	Fructose	80.0	77.0	103.3
Polyols	Manitol	94.6	95.6	103.0
	Sorbitol	95.0	95.7	100.2
	Glycerol	94.1	91.9	106.9
Aminoacids	Glycine	98.4	93.5	101.8
	Alanine	96.0	92.1	99.0
	Leucine	98.2	91.8	103.0
	Histidine	97.5	93.7	97.1
Surfactants	Polysorbate 20	86.0	84.7	76.4
	Polysorbate 80	90.8	88.7	80.3
Polymers	Dextran-40	92.9	92.9	107.8
	PEG 6000	91.0	89.3	99.7
Salts	NaCl	96.4	93.2	90.9
	KCl	93.4	92.6	81.6
	MgCl ₂	95.0	95.0	82.5
Incompatible excipients in Tris buffer	–	97.6	94.3	105.4
	Fructose	92.5	93.1	92.8
	Polysorbate 20	85.2	89.5	97.2
	Polysorbate 80	87.3	90.8	98.3
	PEG 6000	93.7	92.1	107.8

Incompatible excipients are in bold.

^a Area (%) = [(rhEGF1–51 peak area + rhEGF1–52 peak area) after freeze–thawing × 100]/[(rhEGF1–51 peak area + rhEGF1–52 peak area) before freeze–thawing].

^b Purity (%) = [(sum of rhEGF impurity peaks area) × 100]/[(sum total detected peaks area)]. This is for the sample after freeze–thawing cycles.

^c ELISA (%) = [(rhEGF ELISA concentration after freeze–thawing) × 100]/[(rhEGF ELISA concentration before freeze–thawing)].

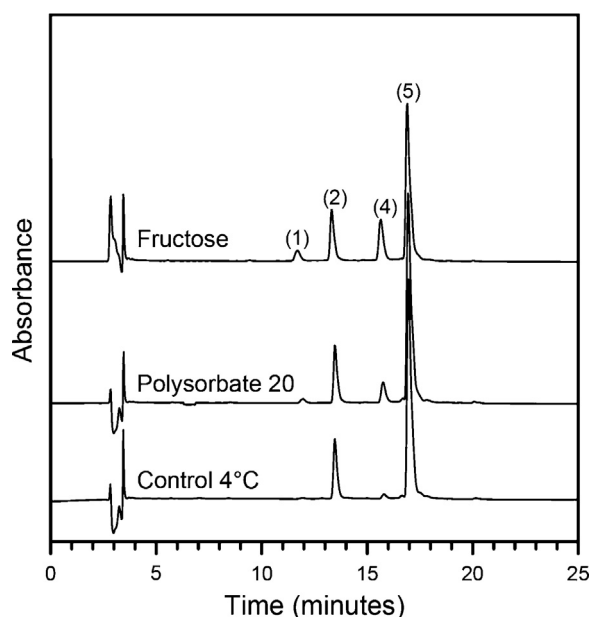


Fig. 8. RP-HPLC profile of rhEGF after three freeze–thawing cycles at -20°C , with two incompatible excipients. Peak (1): early eluting rhEGF1–51 by-product; peak (2): native rhEGF1–51; peak (4): early eluting rhEGF1–52 by-product; peak (5): native rhEGF1–52.

the result also shows an interaction effect among excipients and buffer ions, although some excipients showed a similar detrimental effect.

Representative RP-HPLC chromatograms of the most significant incompatible excipients on rhEGF stability after freeze–thawing are shown in Fig. 8. The control sample after three cycles of freeze–thawing at -20°C was not significantly affected. The sample containing fructose showed a strong increase in peaks 1 and 4. Samples in presence of glucose, polysorbate 20, polysorbate 80 and PEG 6000 also showed a small increase in the same peaks 1 and 4, corresponding with Met-21 oxidation for rhEGF1–51 and rhEGF1–52 as previously characterized (see Table 4). The intensities of these peaks in presence of fructose were much stronger than those obtained with the other excipients. The formation of methionine sulfoxide in Met-enkephalin peptide has been ascribed to the presence of fructose and glucose (Jakas and Horvat, 2008). A strong correlation between Met oxidation and sugar concentration was reported. A higher oxidative potential to damage methionine residues in peptides was reported for fructose. According to our knowledge, the oxidation of proteins by impurities in polysorbates and PEG has been well documented, but not for the use of reducing sugars on protein formulation (Jakas and Horvat, 2008).

4. Conclusions

The chemical degradation of rhEGF showed both acid and basic catalysis with a maximal stability in pH values ranged from 6.0 to 8.0. CD results revealed that this protein was stable in an extensive pH range, keeping the secondary structure very similar to the native one. Aggregation of rhEGF was minimized at pH values ranged from 6.0 to 8.0 as indicated in the SEC-HPLC and light scattering results. Phosphate, Tris and histidine buffers at pH near to 7.0 showed the lower reaction rate constants by RP-HPLC and ELISA methods. Nor the ionic strength, until 2% (w/v) NaCl concentration, neither the rhEGF concentration (25–1000 $\mu\text{g/mL}$) had significant effect on the reaction rate constants. Most rhEGF–excipient instability occurs among this protein and reducing sugars. Polymers like PEG 6000 and polysorbates 20 and 80 increasing Met-21 oxidation. The rhEGF

oxidation and deamidation were the most common degradation pathways. Our results suggest the complexity to achieve long-term stability of rhEGF in a liquid formulation; due to the difficulties in controlling deamidation degradation pathway in solution. That is why a solid state formulation should be explored to find an appropriate technical solution (manuscript in preparation).

Conflict of interest

The work described here has not been published previously and none of the authors have any conflict of interest to declare that limit the publication of this paper in this journal.

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